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VOL. 27, SEC. C. JULY, 1949 NUMBER 4

STUDIES OF CANADIAN THELEPHORACEAE

III. SOME NEW SPECIES FROM BRITISH COLUMBIA[1]

BY H. S. JACKSON AND ELIZABETH RUTH DEARDEN[2]

Abstract

In the present contribution four species of *Peniophora* and five of *Corticium*, collected in British Columbia and presumed to be new to science, are described and illustrated.

Introduction

In connection with a general study of the Thelephoraceae of Canada, the writers have had the privilege of studying a considerable number of collections made in British Columbia mostly by members of the staff of the Dominion Laboratory of Forest Pathology with headquarters at Victoria, B.C. During June and July 1948 the senior author spent several weeks collecting on Vancouver Island, during which period over six hundred specimens were obtained. Among the collections so far studied a number of species have been encountered that are apparently new to science. In the following pages nine such species, four in *Peniophora* and five in *Corticium*, are described and illustrated. Comments on the various species with suggestions as to relationships are furnished following the descriptions.

Description of Species

Peniophora resinosa sp. nov. (Fig. 1)

Fructificatio late effusa, ochraceo-fulva, adnata, 400–550 μ crassa; subiculum ex intertextis et partim paralleliter currentibus hyphis compositum, 3.5–4.5 μ latum, tunicis incrassatis, supra quas sunt hyphae tunicis tenuibus praeditae, nodoso-septatum; cystidia gracilia, clavata, 70–150 × 7.5–12 μ, e subhymenio emergentia et 3/4 longitudinem extrusa, resinosa re supra incrustata, apice obtuso, tunicis irregulariter infra incrassatis; basidia clavata, 30–40 × 6.5–7.5 μ, 4 sterigmata gerentia; basidiosporae late ellipsoideae, 7–9 × 4.5–5.5 μ, tunicis tenuibus, levibus, non amyloideis.

Fructification widely effused on decorticated wood, cream buff, becoming darker with age, adnate, in section 400–550 μ thick, ceraceous, surface minutely porous to hispid due to emergent cystidia, sometimes becoming cracked in

[1] *Manuscript received March 31, 1949.*

Contribution from the Department of Botany, University of Toronto, Toronto, Ont. This study was carried out with the assistance of a grant in aid of research furnished by the University of Toronto.

Nos. I and II of this series appeared in Can. J. Research, C, 26 : 128–139 and 143–147. 1948.

[2] *The writers are indebted to Miss Margaret H. Thomson for the preparation of the Latin diagnoses and to all those who have contributed specimens.*

[The June issue of Section C (Can. J. Research, C, 27 : 45-145. 1949.) was issued July 14, 1949.]

age, margin undifferentiated, determinate, thinning out; subiculum with a basal layer of interwoven, in part horizontal, hyphae, 3.5–4.5 μ broad with thickened walls, upper layer of more or less erect hyphae with thin walls, all with clamps, a considerable amount of resinous material present; cystidia numerous, slender, clavate, 70–150 × 7.5–12 μ, arising in the subhymenium and extruded 3/4 their length, incrusted above with resinous material, apex obtuse, walls thin above, irregularly thickened below, often with capillary lumen; basidia clavate 30–40 × 6.5–7.5 μ, bearing four subulate, slightly arcuate sterigmata; basidiospores broadly ellipsoid, 7–9 × 4.5–5.5 μ, flattened and appearing straight on one side with lateral apiculus, walls thin, smooth, nonamyloid.

Specimens examined:

British Columbia: On *Picea sitchensis*, Queen Charlotte City, Q.C. Isls. Aug. 19, 1944, R. E. Foster, OTB *16063* (*V1711*),* OTB *16030* (*V1714*), OTB *16025* (*V1723*), OTB *16026* (*V1732*) **type**, OTB *16067* (*V1736*), OTB *16068* (*V1929*); on *Pseudotsuga taxifolia*, Cowichan Lake, Vancouver Isl., Aug. 1947, OTB *17618* (*V2736*).

Superficially this species somewhat resembles *P. gilvidula* Bres., based on material collected by J. R. Weir in Montana, but the cystidia and spores of *P. resinosa* are appreciably larger and the former species lacks clamps at the septa. Another species showing superficial resemblance is *P. Weirii* Bres., recorded only from Idaho. That species, however, is described by Burt as having gloeocystidia as well as cystidia, the latter with thin walls. The spores also are much narrower than in *P. resinosa*, being described as cylindrical, 6–8 × 3–3.5 μ.

Peniophora unica sp. nov. (Fig. 2)

Fructificatio late effusa, ceracea, alba vel ochraceo-fulva, 80–150 μ crassa, siccitate vernicosa membrana fit; subiculum e duabus straturis compositum, inferiore stratura e laxe intertextis hyphis altera 2–3 μ diam., superiore e confertis rectis hyphis composita, nodoso-septatum; cystidia late obclavata vel subcylindracea, 55–75 × 12–16 μ, apice obtuso deinde maturitate crasse tunicato; basidia clavata vel cylindracea, 18–20 × 3–4 μ, 4 paene recta sterigmata gerentia; basidiosporae ellipsoideae, 3–3.5 × 2–2.5 μ, laterilater compressae, tunicis tenuibus, levibus, non amyloideis.

Fructification widely effused, ceraceous, white to cream buff, thin, 80–150 μ, adnate, drying to a vernicose film, surface pruinose or minutely hispid due to projecting cystidia; subiculum of two layers of about equal thickness, the lower layer made up of loosely interwoven, nodulose hyphae, 2–3 μ in diameter, the upper layer somewhat obscure but made up of closely compacted upright hyphae, all hyphae with clamps; cystidia broadly obclavate or subcylindrical, rarely fusiform, 55–75 × 12–16 μ, tapering to a narrow obtuse apex, when young thin walled and often with slight incrustations at apex, becoming thick walled and devoid of contents, often with one to four pseudosepta at maturity,

* *The herbarium numbers preceded by the letter V are the numbers of the herbarium of the Dominion Laboratory of Forest Pathology at Victoria, B.C. Those preceded by the letters OTB are in the mycological herbarium of the Division of Botany and Plant Pathology, Department of Agriculture, Science Service, Ottawa. All collections cited are also deposited in the mycological herbarium of the University of Toronto, TRT.*

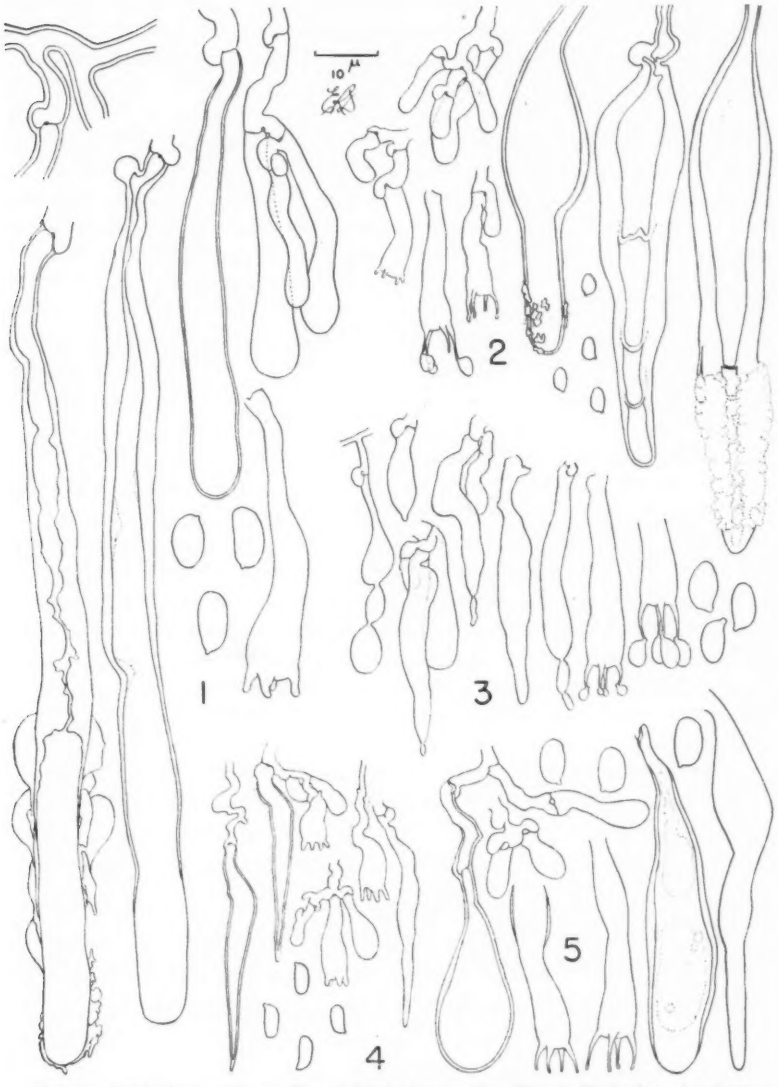

FIG. 1. Peniophora resinosa; FIG. 2. Peniophora unica; Fig. 3. Peniophora inusitata; FIG. 4. Peniophora phlebioides, *all showing cystidia, basidia, and spores*; Fig. 5. Corticium testatum, *showing gloeocystidia, basidia, and spores. Drawn to a uniform scale with the aid of a camera lucida from potassium hydroxide – phloxine mounts, and reproduced at a magnification of approximately 1000* ×.

embedded in the upper layer of the substratum or extending 1/2–1/3 length above hymenium; basidia clavate to cylindrical, 18–20 × 3–4 μ, bearing four slender, nearly straight sterigmata; basidiospores ellipsoid, 3–3.5 × 2–2.5 μ, laterally flattened with minute apiculus, walls thin, smooth, nonamyloid.

Specimen examined:

British Columbia: On wood of *Abies lasiocarpa*, Prince George, P. J. Salisbury, Aug. 27, 1945, OTB *21416* (*V4061*) **type.**

The combination of the ceraceous character of the fructification, together with the large cystidia and minute spores, make this species unique in our experience. Its relationship with other described species is not clear.

Peniophora inusitata sp. nov. (Fig. 3)

Fructificatio resupinata, late effusa, cremeo-ochracea, in superficie membranacea vel pelliculosa, separabilis; subiculum ex hyphis inferioribus in longitudinem, superioribus plus minusve recte dispositis compositum, nodoso-septatis; cystidia 20–45 × 4–5.5 μ, subulata, leviter emergentia, apice moniliformi vel elongatis segmentis praedito; basidia clavata, 20–25 × 5.5–6.5 μ, quattuor sterigmata gerentia; basidiosporae ellipsoideae vel obovatae, 5.5–8 × 3.5–5 μ, tunicis levibus, non amyloideis.

Fructification resupinate, widely effused, separable, hymenial layer cream buff becoming pinkish cinnamon buff, membranous to pelliculose, occasionally rimose exposing the white fibrous lower subiculum, which is made up of longitudinally disposed hyphae of varying width 1.5–3.5–5 μ, with clamps and sometimes slightly enlarged at septa; subhymenial layer of upright, somewhat nodulose hyphae; cystidia 20–45 × 4–5.5 μ arising from the same level as the basidia or slightly below, projecting about one-third their length, subulate, sometimes flexuous, frequently terminated by one or more moniliform swellings or tipped by one or more narrow elongated segments, which may be shed at maturity; basidia clavate, tapering to a narrow base, somewhat flexuous below, often subcylindric above, 20–25 × 5.5–6.5 μ, bearing four slender sterigmata 3.5–5 μ long; basidiospores ellipsoid or asymmetrically obovate, rounded on either side with prominent lateral apiculus, 5.5–8 × 3.5–5 μ, walls smooth, nonamyloid.

Specimen examined:

British Columbia: On wood of branch of *Populus trichocarpa*, Cinema, B.C., W. G. Ziller, Sept. 16, 1948, OTB *21417* (*V3721*) **type.**

The cystidia in this species might properly be interpreted as cystidioles and perhaps are in the nature of modified basidia. The relationship of the species with others is not at all clear.

Peniophora phlebioides sp. nov. (Fig. 4)

Fructificatio effusa, adnata vel 150 μ crassa, alba vel cremea, molliter gelatinea, siccitate dein nonnihil crassa vernicosa stratura; subiculum obscurum, hyphis tunicis gelatineis praeditis, nodoso-septatum; cystidia acicularia, 25–35 × 3.5–4.5 μ, 1/2–2/3 longitudinem emergentia, apicem acuminatum versus angustiora, tunicis tenuibus vel leviter incrassatis, non incrustatis; basidia subcylindracea vel clavata, 12–20 × 3.5–4.5 μ, 4 sterigmata gerentia; basidiosporae cylindraceae, 4.5–5.5 × 2–2.5 μ, tunicis tenuibus, levibus, non amyloideis.

Fructification effused, adnate, up to 150 μ thick, white to cream becoming gray, when fresh soft gelatinous, drying to a somewhat thick vernicose layer, surface somewhat folded and with mucous strands, becoming cracked in age, margin thinning out; subiculum somewhat obscure, made up for the most part of hyphae with gelatinized walls, suberect in the lower part, in places with a more or less horizontal subhymenial layer looser in arrangement than in the lower subiculum; hyphae 1.5–2.5 μ, somewhat nodulose, walls gelatinized, with clamps; cystidia numerous, slender acicular, 25–35 × 3.5–4.5 μ, arising from the subhymenium and emergent 1/2–2/3 their length, gradually narrowing to an acuminate apex, walls thin or slightly thickened, smooth, not incrusted; simple hyphalike paraphyses present in the hymenium; basidia subcylindrical to clavate, somewhat flexuous, 12–20 × 3.5–4.5 μ, bearing four stout, upright sterigmata; basidiospores cylindrical, 4.5–5.5 × 2–2.5 μ, flattened and appearing straight on one side, with lateral apiculus, walls thin, smooth, nonamyloid.

Specimens examined:

British Columbia: On *Pseudotsuga taxifolia*, Swartz Bay, North Saanich, Vancouver Isl., Sept. 23, 1943, I. Mounce, OTB *11523*, *11546*, **type;** New Denver, A. MacKinnon, July 3, 1947, OTB *17616* (*V2721*).

This ceraceous species appears to be amply distinct from others with which it might seem to be related. Bourdot & Galzin would probably have included it in *Corticium* in the group having tapering cystidioles where it would key to *C. subseriale* B. & G. The microscopic characters clearly show that it is neither that species nor the subspecies *C. ochraceo-fulvum* B. & G. Because of the tendency for folds in the hymenium the species approaches *Phlebia*. It is a matter of indifference at present whether such ceraceous species with small cystidia are included in *Corticium* or *Peniophora*. It seems probable that this group, part of which are now in *Corticium* and part in *Peniophora*, will sometime be combined in a separate genus with related forms now in *Phlebia*.

Corticium testatum sp. nov. (Fig. 5)

Fructificatio in elongatis vel fusiformibus areolis consistens, 3–8 × 1–2 cm., ochraceo-fulva, ceracea, adnata, 50–75 μ crassa; subiculum ex intertextis hyphis gelatineis tunicis praeditis compositum, nodoso-septatum; gloeocystidia late clavata vel infra clavato-ventricosa et supra subcylindraceo-flexuosa, 30–55 × 8–12 μ, tunicis tenuibus vel leviter incrassatis; basidia infra ventricosa, supra cylindracea, 30–40 × 4.5–5.5 μ, maturitate supra hymenium extrusa, 4 leviter arcuata 5–6 μ longa sterigmata gerentia; basidiosporae late ellipsoideae, 6–7 × 4–5 μ, tunicis tenuibus, levibus, non amyloideis.

Fructification forming irregularly elongated or fusiform patches 3–8 × 1–2 cm., cream buff, ceraceous, adnate, 50–75 μ thick, margin thinning out abruptly; subiculum made up of fine interwoven hyphae with walls gelatinized, irregularly enlarged at intervals and these enlargements developing to cylindrical or pyriform structures with thick walls that appear irregularly scattered, developing in the hymenium to the more typical gloeocystidia, clamps present throughout; gloeocystidia variable, broadly clavate or clavate-ventricose below and subcylindrical-flexuous above, 30–55 × 8–12 μ, walls thin or the clavate

ones sometimes with walls slightly thickened and indistinctly lamellate, contents guttulate becoming resinous; basidia few in the specimen examined, ventricose below, cylindrical above, 30–40 × 4.5–5.5 μ, extending above the level of the hymenium about one-half their length at maturity, bearing four slightly arcuate, subulate sterigmata 5–6 μ long, walls sometimes slightly thickened below; basidiospores broadly ellipsoid, 6–7 × 4–5 μ, somewhat flattened and appearing nearly straight on one side with lateral apiculus, walls thin, smooth, nonamyloid.

Specimen examined:

British Columbia: On *Pseudotsuga taxifolia*, Cowichan Lake, Vancouver Isl., Mar. 14, 1947, P. J. Salisbury, OTB *21418* (*V2627*) **type.**

The broad gloeocystidia, the walls of which become thickened below, are characteristic and might be interpreted as cystidia. The ventricose basidia, cylindrical above are also characteristic. The species seems amply distinct from other ceraceous gloeocystidiate forms that have been described.

Corticium praeteritum sp. nov. (Fig. 6)

Fructificatio in parvis areolis effusa, 100–200 μ crassa, alba, adhaerens; subiculum hyphis intricatis tenuiter tunicatis, 1.5–3 μ diam., nodoso-septatis; gloeocystidia cylindracea, 30–60 × 4–6 μ, e subhymenio emergentia; aliae structurae quae in hymenio sunt, subpyriformes, vel abrupte globosae, capitatae, 13–20 × 6.5–9 μ; basidia gracilia 25–30 × 5–8 μ, leviter ventricosa, 4 gracilia sterigmata gerentia; basidiosporae cylindraceae vel subellipsoideae, 5–7.5 × 2.5–3.5 μ, tunicis tenuibus, levibus, non amyloideis.

Fructification effused in small areas, submembranous, dry, white, rather thin, 100–200 μ, adherent, margin thinning out, subiculum made up of closely interwoven hyphae, no horizontal basal layer; hyphae for the most part with thin walls, 1.5–3 μ in diameter, not gelatinized, a few are present with thickened walls and others intermixed with fine incrustation, clamps regularly present; gloeocystidia long cylindrical or flexuous cylindrical, arising in the subhymenium, 30–60 × 4–6 μ, sometimes tapering gradually from below, obtuse at apex, not projecting; other structures (modified basidia?) subpyriform or abruptly globose capitate arising in the hymenium, 13–20 × 6.5–9 μ; basidia slender, 25–30 × 5–8 μ, slightly ventricose above the middle, bearing four slender, slightly arcuate sterigmata, 3.5–4.5 μ long; basidiospores cylindrical or subellipsoid, 5–7.5 × 2.5–3.5 μ, flattened and appearing straight on one side with prominent lateral apiculus, walls thin, smooth, nonamyloid.

Specimen examined:

British Columbia: On wood of *Alnus rubra*, Mt. Douglas Park, Vancouver Isl., H. S. Jackson, July 2, 1948, **type** in TRT.

The presence of the subpyriform or globose capitate structures, which may be modified basidia, in the hymenium together with the occurrence of typical gloeocystidia makes this species unique in our experience. The relationship is not clear but obviously not with the "Gloeocystidiales" section of *Peniophora* in the Bourdot & Galzin classification.

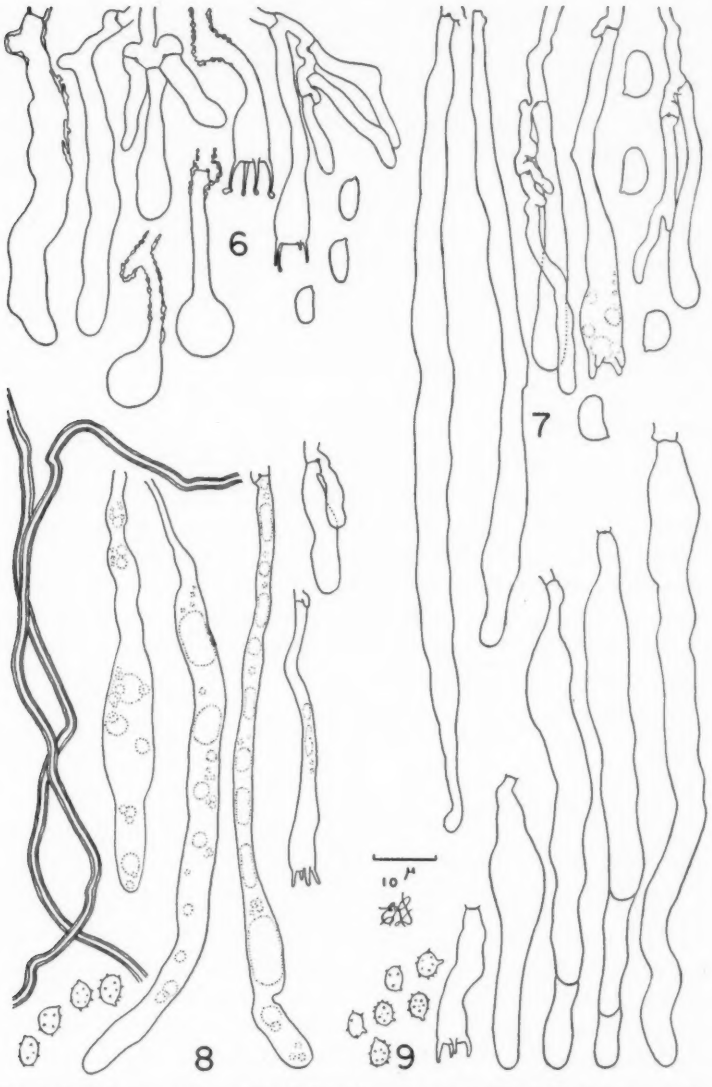

FIG. 6. Corticium praeteritum; Fig. 7. Corticium separatum; Fig. 8. Corticium quaesitum; FIG. 9. Corticium propinquum, *all showing gloeocystidia, basidia, and spores. In Fig. 6 below, are two globose capitate structures from hymenium, and in Fig. 8, to the left, subicular fibrous hyphae. Drawn to a uniform scale with the aid of a camera lucida from potassium hydroxide – phloxine mounts, and reproduced at a magnification of approximately 1000×.*

Corticium separatum sp. nov. (Fig. 7)

Fructificatio late effusa, ceracea, ochracea, adnata, 600–900 μ crassa; subiculum ex erectis nodulosis hyphis compositum, 2–3 μ diam., nodoso-septatum; gloeocystidia cylindracea vel anguste fusiformia, ad apicem obtusa, 65–130 × 4.5–6 μ; basidia graciliter clavata, 45–50 × 5.5–6.5 μ, 4 gracilia subulata 5–5.5 μ longa sterigmata gerentia; basidiosporae oblongae cylindraceae, 7–8 × 3.5–4.5 μ, tunicis tenuibus, levibus, non amyloideis.

Fructification ceraceous, becoming indurated and rimose, ochraceous, widely effused, adnate at first but separable when dry, 600–900 μ thick, margin thinning out to a vernicose film; subiculum made up of upright nodulose hyphae 2–3 μ in diameter, often collapsed, with clamps; gloeocystidia long, cylindrical or narrowly fusiform, subflexuous, 65–130 × 4.5–6 μ, obtuse at apex, arising at different levels in the subiculum and extending to surface of hymenium, not projecting; basidia slenderly clavate, 45–50 × 5.5–6.5 μ, bearing four subulate, slender sterigmata, 5–5.5 μ long; basidiospores oblong cylindrical, 7–8 × 3.5–4.5 μ, flattened and slightly concave on one side, walls thin, smooth, nonamyloid.

Specimen examined:

British Columbia: On *Abies grandis*, Cadboro Bay, Vancouver Isl., Nov. 21, 1940, J. E. Bier, OTB *F10111* **type.**

There are not many truly ceraceous species of *Corticium* having gloeocystidia and all such with which we are familiar except *C. testatum* described above and *C. umbratum* B. & G. have spore walls showing some degree of amyloidity. *C. umbratum*, according to Lundell who transferred the species to *Gloeocystidium*, has gloeocystidia and the spores in available material are not at all amyloid. The species described above differs markedly from *C. umbratum* in gross appearance and in the measurements of both gloeocystidia and spores. The former also has regular clamps at the septa which are absent or rare in the latter.

Corticium quaesitum sp. nov. (Fig. 8)

Fructificatio late effusa, 400–600 μ crassa, margine abrupto, floccosa; subiculum partim e dense intertexta basali stratura fibratarum, crasse tunicatarum, 1.5–2.5μ diam., hypharum quae in solutione Melzer fuscae fiunt compositum, tenue tunicatis hyphis gloeocystidia et basidia supra producentibus; gloeocystidia cylindracea vel clavata, 60–120 × 5.5–8 μ; basidia gracilia clavata, 35–45 × 5–5.5μ, 4 sterigmata gerentia; basidiosporae subglobosae vellate vel ellipsoideae, 3.5–4.5 × 3–3.5 μ, tunicis tenuibus, minute asperulatis, fortiter amyloideis.

Fructification widely effused, cream buff, 400–600 μ in thickness, surface subceraceous, broken into small tufted areolae, exposing the woolly floccose subiculum, margin abrupt floccose; subiculum made up in part of densely interwoven basal layer of fibrous nonseptate hyphae, 1.5–2.5 μ in diameter, with thick walls, turning brown in Melzer's solution; an upper layer of more or less upright thin-walled hyphae with clamps gives rise to the gloeocystidia and basidia; gloeocystidia numerous, cylindrical to clavate, somewhat flexuous, 60–120 × 5.5–8 μ, contents guttulate becoming pseudoseptate after withdrawal of contents; basidia slenderly clavate, 35–45 × 5–5.5 μ, bearing four

upright, subulate sterigmata; basidiospores subglobose or broadly ellipsoid, 3.5–4.5 × 3–3.5 μ; slightly depressed on one side with inconspicuous apiculus, walls thin, minutely asperulate, strongly amyloid.

Specimen examined:

British Columbia: On decayed log of *Pseudotsuga taxifolia*, Nitinat River, Vancouver Isl., Oct. 30, 1946, P. J. Salisbury, OTB *17109* (*V2596*) **type.**

The only collection available is not in the best of condition and the above description of the fructification may need emendment when other material becomes available. The species however possesses such an unusual combination of characters that it seems desirable that it be placed on record. It may be distinguished from all others known to us by the presence in the subiculum of fiber hyphae with pseudoamyloid walls, together with the long slender gloeocystidia and the strongly amyloid spores with minutely roughened walls.

The presence of the pseudoamyloid hyphae in the subiculum serves to relate the species to *Corticium odoratum* (Fr.) B. & G., *C. galactinum* Fr., *C. abeuns* Burt (=*Gloeocystidium ochroleucum* Bres. & Torr.), and *C. praestans* Jackson. As has been previously noted by the writer, this character is suggestive of a possible relationship with *Vararia* (=*Asterostromella*). The rough amyloid spores taken together with the pseudoamyloid hyphae suggest a possible relationship for the species here described to the group of *Vararia* species having similar rough-walled amyloid spores such as *Vararia granulosa* (Pers. ex Fr.) Laurila.

Corticium propinquum sp. nov. (Fig. 9)

Fructificatio late effusa, vel in areolis, cremeo-alba, adnata 125–200 μ crassa, margine abrupto; subiculum ex erectis hyphis compositum, non nodoso-septatum; gloeocystidia flexuosa, cylindracea vel subclavata, 50–90 × 6–8.5 μ, e basi subiculi emergentia; basidia cylindracea, 20–25 × 4.5–5.5 μ, infra ventricosa, 4 paene recta 4 μ longa sterigmata gerentia; basidiosporae subglobosae, 3.5–4 × 3–3.5 μ, apiculo prominente, tunicis tenuibus, verrucosis, fortiter amyloideis.

Fructification broadly effused, or in small irregular areas, creamy white, adnate, 125–200 μ thick, surface soft membranous, becoming rimose, exposing the substratum, margin abrupt; subiculum with a few horizontal hyphae but for the most part with upright, thin-walled, mostly collapsed hyphae without clamps; gloeocystidia numerous, flexuous, cylindrical or subclavate, often ventricose below, 50–90 × 6–8.5 μ, arising from the base of the subiculum or at various levels and extending to the surface of the hymenium, not noticeably projecting; basidia cylindrical, 20–25 × 4.5–5.5 μ, ventricose or subventricose below, bearing four nearly straight or slightly arcuate sterigmata 4 μ long; basidiospores subglobose, 3.5–4 × 3–3.5 μ, with prominent peglike apiculus, walls thin, verrucose, strongly amyloid.

Specimen examined:

British Columbia: On log of *Thuja plicata*, Cowichan Forest Experiment Station, Lake Cowichan, B.C., Sept. 21, 1943, R. E. Foster, OTB *21419* (*V379*) **type.**

The relationship of this species is probably not with *C. quaesitum* described above but with such gloeocystidiate forms having amyloid spore walls as *Corticium porosum* B. & C. and *Gloeocystidium Karstenii* B. & G. It differs from the former in the absence of clamps and from the latter in the smaller rough-walled spores, in gross characters of the fructification, and occurrence on a coniferous substratum.

THE INTERMEDIATE METABOLISM OF *PSEUDOMONAS AERUGINOSA*

I. THE STATUS OF THE ENDOGENOUS RESPIRATION[1]

By Flora C. Norris,[2] Jack J. R. Campbell,[3] and Phyllis W. Ney[4]

Abstract

The endogenous respiration of *Pseudomonas aeruginosa* was shown to differ in several respects from that of other organisms. Although values obtained for the degree of oxidative assimilation of glucose agree with those reported by other workers, this synthesis of storage products was found not to be inhibited by the usual concentrations of 2,4-dinitrophenol. The techniques commonly employed to reduce stored products had no effect on this organism. The endogenous respiration was shown to function normally in the presence of added substrate.

In studies of aerobic microorganisms the influence of the rate of endogenous respiration is of importance in interpreting data relative to metabolism. However, little attention has been paid to the problem of the endogenous respiration of these microorganisms.

The most common approach to the problem of endogenous respiration is to work with cultures whose endogenous activity has been reduced to a point where it is not considered a serious interfering factor. This is usually done by devising a medium that produces cells with good metabolic activity but with practically no storage materials (14). It is not always possible to produce cells with low storage materials, and other means of obtaining cells with reduced endogenous activity, such as aeration of the resting cell suspension or aging at low temperatures, have been employed. Quastel and Whetham, (10) working with *Escherichia coli*, were the first to suggest the starvation technique now commonly used. This technique consists of vigorously aerating a resting cell suspension in non-nutrient solution for two to four hours before use, thus forcing the organisms to oxidize their stored products.

Many workers report that endogenous respiration is suppressed in the presence of a readily oxidizable substrate and thus should be disregarded. Stier and Stannard (12) working with baker's yeast, Clifton (5) with *Pseudomonas calco-acetica*, Clifton and Logan (6) with *E. coli*, Doudoroff (7) with *P. saccharophila*, Bernstein (2) with *P. saccharophila*, and Barker (1) with *Protoheca zopfii* concluded that endogenous respiration is completely inhibited by substrate. On the other hand Reiner, Gest, and Kamen (11), by the use of radioactive carbon, obtained data indicating a stimulation of the endogenous respiration of yeast in the presence of glucose or acetate.

[1] *Manuscript received March 16, 1949.*

Contribution from the Department of Dairying, The University of British Columbia, Vancouver, B.C. This study was carried out under a grant from the National Research Council of Canada, laboratory facilities being found by The University of British Columbia.

[2] *Graduate Student.*

[3] *Associate Professor of Dairying.*

[4] *Formerly Graduate Student, now Junior Bacteriologist, Fisheries Research Board of Canada, Vancouver.*

The present study was undertaken to clarify the status of the endogenous respiration of the obligate aerobe, *P. aeruginosa*, particularly with reference to the influence of oxidizable substrate. This information is an essential prerequisite to a detailed study of the metabolism of this organism.

Methods

The culture of *P. aeruginosa* A.T.C. 9027, used throughout, was a typical, strongly pigmenting strain. A study of the nutrition of the organism as related to pigment production has previously been reported (3, 4, 8).

Stock cultures of the organism were maintained in a liver extract gelatin agar under refrigeration conditions after initiation of growth at 30° C. For use in experimental work the culture was transferred two to three times at 24 hr. intervals in Sullivan's medium (13). In order to maintain a vigorous undissociated culture, a fresh transfer was taken semimonthly from a refrigerated stock. The medium used in growing cells for Warburg experiments consisted of 0.3% ammonium dihydrogen phosphate, 0.1% magnesium sulphate septahydrate, 0.3% carbon source, and 0.5 p.p.m. of iron added as ferric chloride. The medium was brought to neutrality and dispensed in 100 ml. quantities in Roux flasks. After sterilization a 10% solution of dipotassium hydrogen phosphate was added aseptically to a final concentration of 0.3%. The inoculum used was a 24 hr. culture in Sullivan's medium. The cells were harvested after incubation for 18 to 24 hr. at 30° C., washed with half the growth volume of 0.9% saline, and finally made up to the desired concentration. The concentration of cells in the washed suspension, as determined turbidimetrically by a Fisher Electrophotometer, was measured before each experiment.

A conventional Warburg apparatus was used to follow the oxygen uptake and carbon dioxide production of the cell suspensions. In studies of the influence of inhibiting substances on oxygen uptake the cells were incubated 20 min. with the inhibitor prior to the addition of substrate from the sidearm. All Warburg experiments were run at 31° C. The reaction mixture in the cups was pH 7.2.

Cells subjected to the aeration technique were prepared as described above except that aseptic technique was observed throughout. The concentrated cells were aerated vigorously at room temperature while cells that had remained undisturbed at room temperature served as a control. At specified intervals samples of aerated and control cells were removed and their respective respiratory rates determined.

Experimental

Nature of Storage Products

In order to determine the nature of the storage products of *P. aeruginosa*, respiration quotient values were obtained using heavy suspensions of cells that had been grown with glucose, ammonium succinate, or sodium acetate as the sole source of carbon. Glucose and succinate cells were harvested at

24 hr. and brought to 20 times growth concentrations while acetate cells were harvested at 24 and 49 hr. and brought to 40 times growth concentration. Cell suspensions of essentially the same turbidity were obtained in all cases. From Table I it can be seen that identical R. Q. values were obtained in all cases indicating that the storage product of the cells is the same regardless of growth substrate or age and is a compound that is slightly oxidized.

TABLE I

R.Q. OF CELL SUSPENSIONS IN THE ABSENCE OF SUBSTRATE

Growth substrate	Age of cells, hr.	R.Q.
Glucose	24	1.11
Succinate	24	1.12
Acetate	24	1.13
Acetate	49	1.11

Influence of Substrate

In order to determine the influence of concentration of substrate on the endogenous respiration, cells were suspended at 20 times growth concentration and allowed to oxidize 2, 3, 4, and 5 μM of glucose for 70 min. in the Warburg apparatus. These concentrations require 12, 18, 24, and 30 μM oxygen respectively for complete oxidation. A similar experiment was set up using 6, 9, 12, and 15 μM of acetate (equivalent to 12, 18, 24, and 30 μM oxygen). If the endogenous respiration is disregarded there is found to be a variation of 40% in the degree of oxidation of a given substrate depending upon its concentration and, with the lower concentrations of substrate, the oxygen uptake is shown to be greater than is theoretically possible. If, on the other hand, the endogenous respiration is considered to function normally in the presence of substrate, and the oxygen uptake due to the substrate dissimilation is determined by subtracting the endogenous oxygen uptake from the total uptake, the degree of oxidation is found to be comparable for all substrate concentrations. Moreover, the extent of oxidation is in agreement with the data reported in the literature for related organisms. According to the concept of oxidative assimilation proposed by Barker (1) and Clifton (5), microorganisms oxidize a portion of the energy source to carbon dioxide and water and assimilate the remainder directly into cellular material. The figures usually reported are approximately two-thirds oxidation and one-third assimilation, which is the ratio obtained with *P. aeruginosa* if oxygen uptake due to endogenous respiration is subtracted from the total (Table II).

In order to determine the influence of concentration of cells on amount of oxygen taken up in the presence of a constant amount of substrate, cells harvested from the glucose medium were resuspended at 10, 20, and 40 times growth concentration. The activity of these cell suspensions was determined in the Warburg respirometer with 5 μM of glucose as substrate. If

TABLE II

OXIDATIONS OF GLUCOSE-GROWN CELLS ON VARYING CONCENTRATIONS OF GLUCOSE AND ACETATE

Warburg substrate	Conc. in cup, μM	Total O_2 uptake, μl.	Theoretical uptake, μl.	Percentage of theoretical	O_2 uptake (endogenous subtracted), μl.	Percentage of theoretical (endogenous subtracted)
	Nil	152				
Glucose	2*	336	270	124	184	68
	3	425	402	106	273	68
	4	505	535	92	353	66
	5	565	672	84	413	61.5
	Nil	153				
Acetate	6**	354	270	131	201	74.5
	9	453	402	113	300	75
	12	530	535	99	377	70.5
	15	625	672	93	470	70

* *22 hr. cells.*
** *26 hr. cells.*

the endogenous respiration has been repressed, the same total oxygen uptake should be obtained in all cases since the same amount of substrate is being oxidized by each of the above cell suspensions.

The results recorded in Table III show, however, that the total oxygen uptake increases proportionately with an increase of endogenous respiration. If, on the other hand, the endogenous respiration is subtracted, the percentage

TABLE III

OXIDATION OF 5 μM GLUCOSE BY CELL SUSPENSIONS OF VARYING CONCENTRATION

Concentration of cells	O_2 uptake, endogenous, μl.	O_2 uptake 5μM Glucose		
		Total, μl.	Minus endogenous, μl.	Percentage of theoretical uptake
10×	37	420	383	57
20×	95	540	445	66
40×	200	645	445	66

oxidation is found to be relatively constant in all cases (Table III). The lower value obtained with cells concentrated 10 times is explained by the fact that the curve for these cells had not leveled off at 80 min.

The data in Tables II and III show conclusively that the endogenous respiration of *P. aeruginosa* is unaffected by the presence of oxidizable substrate. It follows that in determining the oxygen uptake on a given substrate, the endogenous respiration must be subtracted from the total uptake.

Influence of Aeration and Storage

In studies on the influence of aeration and storage on endogenous respiration, 24 hr. glucose-grown cells were resuspended in saline at 10 times growth concentration and the suspension divided into two portions. One portion of the suspension was aerated vigorously at room temperature while the other half, serving as control, was allowed to stand undisturbed. Samples of aerated cells were removed at intervals of two, five, and eight and one-half hours while samples of control cells were removed at zero, two, five, and eight and one-half hours. The endogenous activity and the ability to oxidize glucose were determined. The purity of suspensions was checked by Gram stain at the conclusion of the experiment.

Since results similar to those obtained after aeration for eight and one-half hours were also obtained at two and five hours, curves depicting the results for only zero and eight and one-half hours are recorded in Fig. 1. From these

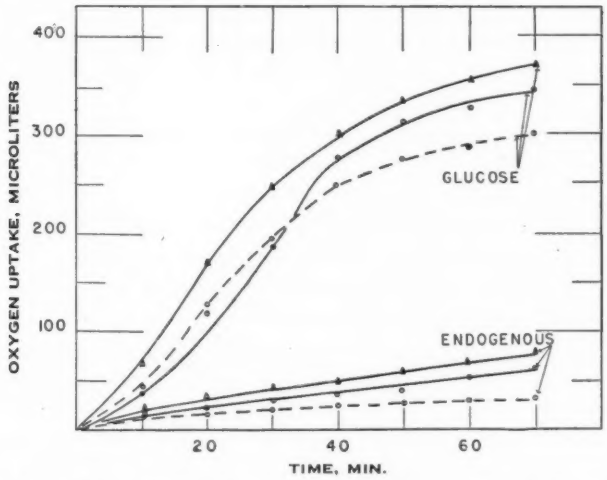

FIG. 1. *The effect of aeration on resting cells of P. aeruginosa. Warburg cups contained 0.5 ml. of cell suspension, 1.5 ml. of M/15 phosphate buffer, and 0.2 ml. of substrate. Final volume was 3.0 ml.*

△——————△ = *Unaerated cells, zero hours.*
○——————○ = *Aerated cells, eight and one-half hours.*
○- - - - - - - - - -○ = *Control cells, eight and one-half hours.*

data it can be seen that there is no significant decrease in the endogenous respiration nor is there any appreciable decrease in the ability of the cells to oxidize glucose after aeration for eight and one-half hours. The slight decrease in activity at eight and one-half hours cannot be attributed to aeration since control cells showed a similar slight decrease. The use of aeration is a recognized procedure for reducing the endogenous respiration

of microorganisms. The inability of this technique to affect the autorespiration of *P. aeruginosa* indicates that there may be something unique about the storage products of the organism.

Additional data on the influence of storage conditions were obtained when a cell suspension was stored in the refrigerator and samples removed daily for study. The endogenous respiration and ability to oxidize glucose were determined. Results recorded in Table IV show that after four days' storage there was no change in the activity of the cells.

TABLE IV

STORAGE OF CELLS

Time of storage, days	Oxygen uptake at 70 min.	
	Endogenous, μl.	3μM glucose, μl.
0	60	290
1	53	310
2	57	294
3	53	312
4	57	285

Inhibition of Synthesis

Since *P. aeruginosa* appears to be exceptional in its behavior when aerated and since it has been shown to possess a mechanism for the oxidative assimilation of substrates, it was considered of value to determine the action of compounds that inhibit synthetic processes.

Sodium azide and 2,4-dinitrophenol have been used extensively as inhibitors of oxidative assimilation (5, 6, 7). Solutions of sodium azide at final concentrations of $M/200$, $M/400$, and $M/1000$ and 2,4-dinitrophenol at $M/1000$, $M/1500$, $M/2000$, M/2500, $M/4000$, and $M/8000$ were used in Warburg experiments with glucose as substrate. In the calculation of these results the respective endogenous respiration values were subtracted from those obtained in the presence of glucose. The presence of dinitrophenol produced no inhibition of oxidative assimilation in any of the concentrations used. $M/1000$ azide, however, increased the oxidation from 64% to 90% of the theoretical value. Higher concentrations of azide inhibited both rate and total amount of oxygen uptake.

Discussion

From this study it is evident that one cannot make generalizations regarding the endogenous respiration of microorganisms since the endogenous respiration of *P. aeruginosa* has been shown to differ in several important respects from that reported for other organisms. Although data obtained using azide as an

inhibitor of oxidative assimilation agree with those reported in the literature for other organisms, 2,4-dinitrophenol, the most common reagent employed for this purpose, failed to inhibit the oxidative assimilation of *P. aeruginosa*. It was also found that the endogenous respiration of this organism could not be reduced by either vigorous aeration or storage of the organism at low temperatures, indicating a possible peculiarity in the nature of the storage products elaborated or the destruction by this technique of certain enzymes or coenzymes associated with the utilization of this storage product. The conclusion that the endogenous respiration functions normally in the presence of added substrate and thus should be substracted in the calculation of the degree of oxidation of the substrate contradicts the practice followed by most workers.

It is possible that the type of endogenous respiration exhibited by *P. aeruginosa* is common to organisms that do not have a constitutive enzyme system for oxidizing glucose (9). The lack of such enzymes indicates that the storage products are not oxidized via glucose or closely related compounds. The enzymes required for the oxidation of stored products thus would not compete with those involved in the oxidation of the substrate.

Much of the confusion and many of the contradictory results reported in the literature may be explained by the fact that there is considerable variation in the nature of the endogenous respiration of various microorganisms. The significance that is to be attached to the rate of endogenous respiration in interpreting data on the intermediary metabolism of microorganisms is thus shown to be of paramount importance.

Acknowledgment

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THE INTERMEDIATE METABOLISM OF *PSEUDOMONAS AERUGINOSA*

II. LIMITATIONS OF SIMULTANEOUS ADAPTATION AS APPLIED TO THE IDENTIFICATION OF ACETIC ACID, AN INTERMEDIATE IN GLUCOSE OXIDATION[1]

By Jack J. R. Campbell,[2] Flora C. Norris,[3] and Margaret E. Norris[4]

Abstract

Acetic acid has been isolated as an intermediate compound in the oxidation of glucose or α-ketoglutaric acid by *Pseudomonas aeruginosa*. Cells of this organism produced under conditions of intense aeration were found to have lost the ability to oxidize acetic acid and this compound was found in large quantities in the growth medium. When these cells were used to carry out the oxidation of glucose, it was found that the oxygen consumed was the amount needed to convert glucose to acetic acid, thus confirming the role of acetate as an intermediate in glucose oxidation. In contrast to the above-mentioned criteria the technique of simultaneous adaptation ruled out acetic acid as a possible intermediate in the oxidation of either glucose or α-ketoglutaric acid. The status of the theory of simultaneous adaptation is discussed in relation to the data obtained.

Progress towards a fuller understanding of the anaerobic metabolism of bacteria has been very rapid during the past twenty years. In contrast little advancement has been made toward the elucidation of the path by way of which bacteria oxidize carbohydrates. Certain valuable contributions such as Lipmann's (4) analysis of the pyruvate oxidizing system of *Lactobacillus delbrueckii* and Barron's (1, 2) studies on the oxidations of the gonococci and streptococci have been made. However, the majority of such studies on oxidation have been concerned with a minor phase of the metabolism of a fermentative organism. Information concerning the carbohydrate metabolism of an obligate aerobe such as *Pseudomonas aeruginosa* is meager. Pervozvanskii (8), by the use of vigorous aeration, and high quantities of substrate in the presence of calcium carbonate, was able to block the aerobic metabolism of fluorescing organisms in the earlier phases and thereby cause large quantities of gluconic and ketogluconic acids to accumulate in the medium. The production of the latter compound under these vigorous conditions has also been demonstrated for *P. aeruginosa* A.T.C. 9027 (6). A further step in the breakdown of glucose was indicated when Lockwood and Stodola (5) showed that 16 to 17% yields of α-ketoglutaric acid were obtained if the oxidation of 2-ketogluconic acid by *Pseudomonas fluorescens* was allowed to continue until the medium had lost all reducing power. Since the conditions to which the organism was subjected during these large scale fermentations were so far removed from those of its normal environment, it is impossible

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[2] *Associate Professor of Dairying.*

[3] *Graduate Student.*

[4] *Senior Student.*

to conclude from these data that the acids produced are normal intermediates in glucose dissimilation. However, they are indicative of the mechanism available to the cell.

Since *P. aeruginosa* has been shown to have an adaptive system for the oxidation of glucose (6), it was thought that it would be a simple matter to eliminate or identify the compounds intermediate in the dissimilation of glucose by the use of the technique of simultaneous adaptation as proposed by Stanier (9). However, when cells were harvested from a growth medium in which glucose was the source of energy, and tested on postulated intermediate compounds, it was found that, with the exception of gluconic acid, a period of adaptation was required before the cell could oxidize any of the substrates. According to Stanier's interpretation, this would mean that of the 50 substances tested only gluconic acid could be an intermediate. Since this list included such compounds as 2-ketogluconic, pyruvic, and acetic acids, the criterion adopted for the elimination of these compounds as possible intermediates would appear to be questionable.

The object of the present investigation was to attempt to identify acetic acid as an intermediate in the oxidation of glucose by *P. aeruginosa* and to check on the soundness of the technique of simultaneous adaptation.

Methods

The methods used in the present study are in a large measure the same as those recorded previously (7). *Pseudomonas aeruginosa* A.T.C. 9027 was used throughout. Any variations in the methods employed are detailed below. The medium used in growing cells for Warburg experiments consisted of 0.3% ammonium dihydrogen phosphate, 0.1% magnesium sulphate septahydrate, and 0.5 p.p.m. of iron added as ferric chloride. The medium was brought to neutrality before autoclaving. After sterilization, 0.3% of an autoclaved 10% solution of dipotassium hydrogen phosphate and glucose, α-ketoglutarate or acetate that had been sterilized by passage through a sintered glass filter, were added aseptically.

In order to isolate a quantity of volatile acid adequate for identification purposes the following procedure was carried out for both glucose and α-ketoglutarate. Two liters of media were prepared as previously described and one liter of this was held as the uninoculated control while the other was dispensed in Roux flasks in 100 ml. quantities. The 10 flasks were inoculated and then incubated at 30° C. for 18 hr. At the end of this time the cells were centrifuged down, the supernatant acidified with sulphuric acid, and two liters of distillate collected by steam distillation. The distillate was neutralized, concentrated on a hot plate to about 150 ml., reacidified with sulphuric acid, and about 130 ml. collected by slow distillation. The liter of uninoculated medium, which contains substrate and phosphate, was treated similarly with the exception that the cells from 100 ml. of the inoculated medium were added before distillation to ensure the presence of at least as much protein as was

present in the liter of supernatant obtained from the inoculated media. The distillates from these controls were alkaline to bromthymol blue; the procedure was thus not carried beyond the initial distillation. Duclaux constants were run on the distillates from the inoculated media. The procedure was as follows: 25 ml. of sample was set aside while exactly 100 ml. was placed in a 250 ml. distilling flask equipped in the usual manner for such determinations. After discarding the first 10 ml. of distillate the subsequent three 25 ml. fractions were collected. These three samples plus the original 25 ml. aliquot were then titrated using a Beckman potentiometer. Three end points were taken for each sample—pH 6.0, 7.5, and 8.5. Essentially the same constants were obtained at each of the end points. Titrations required from 3 to 12 ml. of $N/400$ sodium hydroxide. Standards of formic, acetic, and propionic acids of about the same concentrations as the unknowns were also run.

Experimental

According to the theory of "simultaneous adaptation" cells harvested from a medium containing a specific energy source will attack this substrate or any of the compounds that are intermediate in its breakdown, and therefore present during growth, without a period of adaptation. If acetic acid is an intermediate in the oxidative breakdown of glucose by *P. aeruginosa*, cells of this organism harvested from a glucose medium and tested in a Warburg respirometer should immediately oxidize acetate at a maximum rate. From Fig. 1 it can be seen that this is not the case and therefore, on the basis of the theory of simultaneous adaptation, acetic acid would thus be ruled out as an intermediate in glucose oxidation by this microorganism. When α-ketoglutaric acid was the sole energy source for growth, it was shown that resting cells harvested from this medium also went through a period of adaptation when placed in a Warburg cup with acetate as substrate. However cells that had been harvested from a medium in which acetate was the only source of carbon did oxidize this compound vigorously and immediately, Fig. 1.

In order to determine whether or not acetate or at least some volatile acid was formed during the oxidation of glucose or α-ketoglutarate, cells were harvested from the liquid media containing one or other of these carbon sources and allowed to oxide their parent substrate in 125 ml. Warburg cups. The reaction mixture, which had a total volume of 30 ml., contained 9 mgm. of substrate. The oxidations were stopped by the addition of sulphuric acid and the contents of the flask subjected to steam distillation. Titrations of the distillate with $N/100$ sodium hydroxide are shown in Table I. In Experiment V an atmosphere of 10% air and 90% nitrogen was used in the hope that intermediate compounds would accumulate because the limiting oxygen prevented their further oxidation. However, the results were similar to those of the four experiments run in an atmosphere of air. In every case, small amounts of volatile acid were formed during the oxidation of glucose or α-ketoglutarate by resting cells of *P. aeruginosa*.

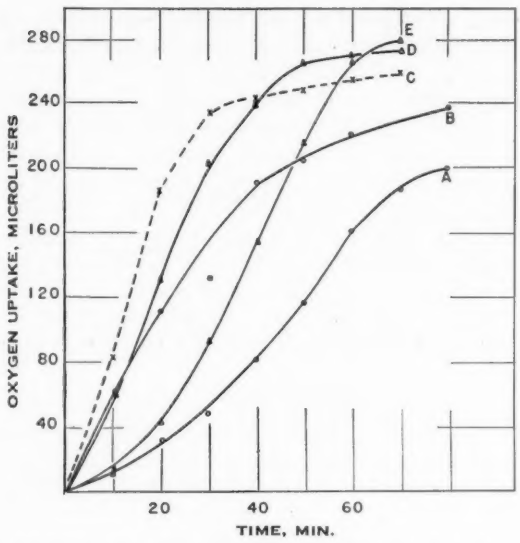

FIG. 1. *Adaptation of glucose and α-ketoglutarate-grown cells to the oxidation of acetate. Warburg cups contained 0.5 ml. of cell suspension; 1.5 ml. of M/15 phosphate buffer, pH 7.2; 0.2 ml. of substrate. Final volume was 3.0 ml. Theoretical oxygen uptake for complete oxidation of any substrate was 403 microliters. Warburg substrates; A, C, and E, acetate; B, α-ketoglutarate; D, glucose. Value of endogenous respiration has been subtracted.*

○———○ = *α-Ketoglutarate-grown cells.*

△———△ = *Glucose-grown cells.*

×- - - - - - - - -× = *Acetate-grown cells.*

TABLE I

PRODUCTION OF VOLATILE ACID BY RESTING CELL SUSPENSIONS

Experiment	Substrate	Ml. of *N*/100 NaOH to neutralize distillate when reaction stopped at		
		10 min.	15 min.	60 min.
I	Endogenous	—	1.25	—
	(α-ketoglutarate)	1.55	1.40	—
II	Endogenous	—	1.10	—
	(glucose)	1.80	1.85	—
III	Endogenous	—	1.30	—
	(glucose)	—	1.85	—
IV	Endogenous	—	1.25	—
	(glucose)	—	2.95	—
V	Endogenous	—	—	1.25
	(glucose)	—	—	1.50

In an effort to identify this volatile acid the supernatant from a liter of 18 hr. culture, in which glucose or α-ketoglutarate had been the only source of carbon, was acidified and steam distilled. Duclaux constants were determined for the concentrated distillates and compared to the values obtained for comparable concentrations of formic, acetic, and propionic acids. Repeated determinations gave similar findings. The results for the distillates of the growth media are seen to parallel those obtained for acetic acid. The constants for acetate differ from those usually reported in the literature but apparently they are an idiosyncrasy of the apparatus or more probably of the dilute solutions used, since the constants usually given are for much more concentrated solutions. Under any circumstance this idiosyncrasy can be used as a criterion for showing definitely that the volatile acid formed during growth is almost solely acetic acid.

TABLE II

DUCLAUX CONSTANTS OF VOLATILE ACID PRODUCED DURING GROWTH

	Standards			Distillate from glucose medium	Distillate from α-keto-glutarate medium
	Formic acid	Acetic acid	Propionic acid		
Fraction A	0.1250	0.2486	0.2860	0.2447	0.2183
Fraction B	0.1526	0.2322	0.2523	0.2380	0.1940
Fraction C	0.2300	0.2612	0.2374	0.2760	0.2360

Further evidence that acetate is an intermediate in glucose oxidation was obtained when it was found that cells harvested after four days' growth in an aerated liquid medium had almost completely lost their ability to oxidize acetic acid. It follows therefore that if acetate is an intermediate it should accumulate under these conditions of growth. This was found to be the case and about 30 times as great a concentration of acetic acid was found in the supernatant from cells that had lost their ability to oxidize acetic acid as in the supernatant from cells that still had the ability to oxidize this compound.

Since one-third of the oxygen consumed during the degradation of glucose would be used in converting acetic acid to carbon dioxide and water, if acetate is an intermediate, cells that cannot oxidize acetate should use only two-thirds as much oxygen as cells that can oxidize this compound. This was found to be true (Table III). The average value for normal cells is the value obtained from the data presented previously (7).

From Table III it can be seen that when glucose is oxidized by cells that have lost the ability to attack acetic acid, the amount of oxygen consumed is almost exactly the amount necessary to convert glucose to acetate, thus confirming the role of this compound as an intermediate in glucose oxidation.

TABLE III

OXIDATIONS BY CELLS UNABLE TO ATTACK ACETATE

	Substrate	Oxygen uptake, μl.
Average value, normal cells	Glucose	273
Observed value for cells unable to oxidize acetate	Glucose	179
Calculated value for cells unable to oxidize acetate	Glucose	182
Observed value for cells unable to oxidize acetate	Acetate	22

Discussion

Yudkin (10) explained enzyme adaptation with the theory that "all examples of enzyme production are cases of increase in enzyme and none are instances of the formation of completely new enzyme" and further that "it is clear that the adaptive enzyme is produced from a precursor or precursors . . . . it is assumed that an equilibrium exists between such precursors and the formed enzyme". According to Yudkin the combination of the enzyme with substrate would result in a disturbance of the equilibrium and more enzyme would be formed from the precursor in order to restore it. It therefore follows that within the physiological limits of the organism, an increased amount of substrate would result in the production of a greater amount of enzyme. This deduction is supported by the work of Kertesz (3) who found this to be true of invertase production by *P. glaucum*. Stanier's theory of simultaneous adaptation contradicts this work for it assumes that when a substrate is attacked by an adaptive enzyme the same amount of enzyme will be produced regardless of the amount of substrate present in the growth medium so that when cells are transferred from conditions where the compound in question is a metabolic intermediate, and therefore present in only very small amounts, to conditions where the same compound is present as parent substrate and in much larger quantity, no increase in amount of enzyme will occur.

In our studies it was found that, of the intermediates that theoretically should be identifiable by simultaneous adaptation, only those that were probably separated by only one enzyme reaction from the parent substrate reacted in accordance with Stanier's predictions. That is, cells grown on a glucose medium could attack gluconic acid without a period of adaptation, but there was a period of adaptation before they attacked 2-ketogluconic acid. Cells grown on pyruvic acid would attack acetic acid without a period of adaptation but cells grown on glucose had a period of adaptation before either pyruvic or acetic acids were oxidized.

Another limitation to the theory of simultaneous adaptation is that, if a pair of compounds are in enzymatic equilibrium, growth on a medium containing either of the compounds results in the production of cells that can immediately attack both compounds. The same examples as discussed above

will serve to illustrate this point. Cells harvested from a glucose medium are found to oxidize gluconic acid immediately at maximum rate and cells harvested from a gluconic acid medium were also found to attack glucose at maximum rate. According to the theory of simultaneous adaptation, g ucose would thus be an intermediate in gluconic acid oxidation. Similar results were obtained for cells grown on either pyruvic or acetic acids when they were tested on either pyruvate or acetate and, according to the theory of simultaneous adaptation, one would be forced to conclude that acetic acid was oxidized by way of pyruvate.

Acknowledgment

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AN IRREVERSIBLE GENE-INDUCED PLASTID MUTATION[1]

By T. J. Arnason[2] and G. W. R. Walker[3]

Abstract

When plants of a variegated barley are self-pollinated, they produce few variegated and many albino offspring. In different years the proportion of albino plants has ranged from 80.2 to 93.1% of the total population. Seed from heads having much green tissue gave rise to a much larger proportion of variegated plants than did seed from heads with more white tissue. Maternal inheritance of plastids is probably the cause of this difference. In crosses F_1 plants are green, variegated, or albino if the ♀ parent is variegated, but if the ♀ parent is green all the progeny are green. The albino plastids thus apparently do not mutate back to normal in the presence of the normal gene. In some F_2 populations deviation from a ratio of 3 green : 1 others is insignificant, in other populations significant deviations, attributed to irregularities of plastid mutation and segregation, occur. F_3 results support the hypothesis that a single pair of genes affecting plastids is segregating in hybrids. The normal (green) gene is dominant if "green" proplastids are present in the egg but not dominant if the proplastids are all "white". From cytological observations on sperms and eggs as well as from the genetic results, it is considered likely that direct plastid transmission to zygotes is exclusively from the female parent.

Introduction

Since plastids can reproduce their kind they may be able to maintain, to a limited degree, independence of nuclear genes. Such independence is shown when there is maternal inheritance of mutated plastids. In a strain of variegated barley described earlier (1) and in the present paper, maternal inheritance of mutated plastids occurs. Inheritance is complicated, however, by continuing mutation of green to albino plastids in the presence of certain nuclear genes. Consequently selfed plants of the variegated strain produce no green offspring, even though eggs formed in green tissue patches must often contain "green" proplastids only. In crosses with green varieties, some segregation ratios are distorted by irregular plastid mutation and plastid segregation.

Plastid Distribution in Variegated Plants

1. *Leaves*

Freehand sections of variegated leaves were prepared by a modification of Froier's (2) method. The freshly cut sections, 20–50μ thick, were examined immediately in a water mount. The addition of a small drop of iodine to each preparation increased the visibility of plastids.

In green mesophyll cells of variegated leaves, 37 to 46 green plastids, 5–7μ in diameter, were counted (Fig. 1). In a transition zone between green and white tissues the number of green plastids was reduced to 1 to 21, and some of the plastids were also reduced in size. Cells with the number of normal plastids reduced contained 28 to 36 small bodies most of which had a diameter

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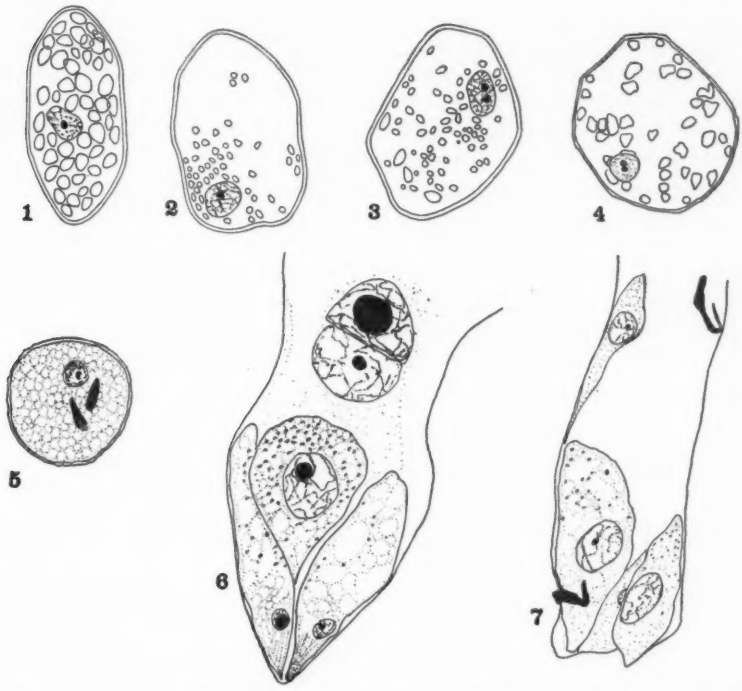
Contribution from the Biology Department, University of Saskatchewan, Saskatoon, Sask.
[2] *Associate Professor of Biology.*
[3] *Research Assistant.*

of 1–3.5μ. The staining reaction of these granules with iodine was similar to that of normal plastids. A cell having four green but slightly reduced plastids and a large number of the small granules is drawn in Fig. 3.



FIGS. 1 TO 7. *All 540×. Camera lucida drawings.* FIG. 1. *Normal green plastids, in outline, in mesophyll of barley leaf.* FIG. 2. *Small mutant plastids in white mesophyll cell of variegated leaf.* FIG. 3. *Mutant and green plastids in cell of variegated leaf.* FIG. 4. *Mutant plastids enlarged after detached leaf had been floated for 24 hr. in 10% sucrose solution.* FIG. 5. *Mature pollen grain containing two sperms.* FIG. 6. *Egg apparatus and fusing polar nuclei in barley embryo sac. Many proplastids in egg.* FIG. 7. *Sperm nucleus lying over egg, 24 hr. after pollination.*

Except at some margins adjacent to green tissue no normal green plastids were observed in "white" cells but the number of small granules was increased, at least in some cells, to 40 to 50 per cell (Fig. 2). The small granules are doubtless mutated plastids, since, when white leaves are floated for 24 hr. on 10% cane sugar solution, they enlarge and deposit starch (Fig. 4).

If the cell lineage of adjacent cells in barley leaf cross sections were traced, one might find that many cell divisions had intervened between the common cell ancestor and the derived mature adjacent cells. For this reason one should not conclude that, if adjacent mesophyll cells differ markedly in plastid content, the cause must be something other than plastid segregation.

2. *Gametes*

Unfertilized eggs in embryo sacs contain, in the cytoplasm, many (more than one hundred) small round bodies that are probably proplastids. These are somewhat less abundant in synergids. No mature plastids are present.

It would be of interest to determine cytologically whether, at fertilization, a naked sperm nucleus enters the egg, or whether sperm cytoplasm enters also. Although sperm cells are known to be formed in male gametophytes of some Angiosperms (3), there is some doubt as to when the cell membrane bounding the individual sperms disappears. Decisive cytological evidence that sperm cytoplasm enters eggs appears to be lacking.

Sperms in pollen grains of barley have been studied in thin sections (7–10μ), stained in various ways. Nearly all the sperms appear to consist of naked, elongated nuclei. A slight "tail" is, however, visible at one end of some sperms, outside the deeply-staining nuclear mass (Fig. 5). Whether this lightly staining portion is cytoplasm or karyolymph is in doubt. No trace of sperm cytoplasm could be distinguished in sperms found near the egg after growth of pollen tubes (Fig. 7).

Phenotypic Segregation in Progenies of Selfed Variegated Plants

In each year since 1942 over 500 offspring of selfed variegated plants have been classified according to phenotype as albino, variegated, or green. Over 80% (80.8 to 93.1%) of each population has consisted of albinos; the remaining plants, making up 6.9 to 19.2% of the total population have been, with few doubtful exceptions, variegated. Of a total of 7381 plants, 6488 (87.9%) were albino, 871 (11.8%) were variegated, and 22 (0.3%) were listed as green. All but one of the green plants were recorded in 1942, the classification being done on young plants with few leaves. In the last three years seedlings classified initially as green have been re-examined at intervals during the season and all of them have shown traces of variegation in later stages of growth. The green plants recorded in 1942 may have been, like these, faintly variegated.

The plants listed as variegated are by no means a uniform group; phenotypically there is a continuous range from white with a faint green stripe in one or two leaves to green with a narrow white stripe in some leaves. If, as was suggested earlier (1), nuclear genes are the same in the variegated and albino plants, then the phenotypic differences may most reasonably be attributed to plastid mutation and plastid segregation. The plastid or proplastid constitution of the fertilized egg will then be expected to have important effects on the phenotype of the leafy plant that develops from it. If the egg contains albino plastids only, the seedling is expected to be albino; If the plastids are mixed, or all green but susceptible to the mutagenic effects of the nuclear gene, the seedlings are expected to be variegated.

On the supposition that ovary and ovule tissues are probably white if the enclosing glumes are white, glumes of variegated heads were marked and the enclosed seeds tested. Results are recorded in Table I. The low proportion

TABLE I

NUMBERS OF ALBINO AND VARIEGATED SEEDLINGS FROM SEEDS FORMED IN WHITE AND VARIEGATED PORTIONS OF THE HEAD RESPECTIVELY

Glumes	Seed number	Seedlings	
		White	Variegated
White	99	98	1
Variegated	319	310	9

of variegated seedlings from the variegated portions of marked heads is doubtless a result of selecting, for glume marking, heads with higher than average amounts of white tissue.

To study differences between head progenies of variegated plants, heads were marked according to the percentage of white awns, the awns being used as an index of the amount of chlorophyll tissue in the heads. The results, recorded in Table II, are believed to be instructive. Heads having large

TABLE II

PROGENY OF INDIVIDUAL HEADS MARKED FOR PERCENTAGE OF WHITE AWNS

% White awns	No. of heads	Seedlings		
		Albino	Variegated	% Variegated
65 – 100	9	100	4	3.8
35 – 64	7	113	17	14.1
0 – 34	8	170	78	31.5

amounts of green tissue produce a much higher proportion of variegated offspring than do those that have less green and more white tissue. Direct plastid inheritance is the most probable cause of the observed differences. Eggs arising in green tissue inherit green plastids, some of which mutate prior to seedling emergence, thus giving rise to variegated plants. Eggs arising in albino tissue have "white" plastids only and give rise to albino plants. Although eggs arising in green tissue would be expected frequently to have all green plastids, no full green offspring are produced. Thus plastid mutation from green to white probably continues to occur prior to seedling emergence in every generation.

Crosses Between Variegated and Green Regal Barley

As previously reported (1) Regal (green) ♀ × variegated ♂ results in all green F_1 (one reported exception) while from the reciprocal cross green, variegated, and albino offspring are produced in no definite proportion. F_1 results from several recent crosses including backcrosses are reported in Table III.

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TABLE III

F_1 PHENOTYPES FROM CROSSES AND BACKCROSSES INVOLVING VARIEGATED (V) AND GREEN (G) PARENTS

Cross	F_1 AND BACKCROSS PHENOTYPES		
	Green	Variegated	Albino
G ♀ × V ♂	55	0	0
V ♀ × G ♂	56	43	151
V F_1 (V ♀ × G ♂) × G	13	0	2
V F_1 (V ♀ × G ♂) × V	8	0	1

The differences between F_1 populations from the reciprocal crosses may most reasonably be explained on the assumption that direct plastid inheritance (maternal) may determine the phenotype. Since there is a large number of albino F_1, one may doubt that any albino plastids are converted to green by the genes contributed by the Regal parent. Albino plants could not be checked for hybridity since they all died in the seedling stage. That the variegated F_1 are hybrids is attested by their green F_2 offspring.

The F_2 results from the Regal ♀ × variegated cross (Table IV) show a good fit to a 3 green : 1 of others (P = .3 to .5). In the reciprocal cross, however, the fit to a 3 : 1 ratio is poor (P is less than .05), there being a considerable excess of variegated and albino segregates. Direct plastid inheritance is the most probable cause of the distorted ratio.

TABLE IV

F_2 SEGREGATION OF HYBRIDS FROM CROSSES BETWEEN REGAL (G) AND VARIEGATED (V) BARLEY

Cross	F_1 phenotypes	No. of F_1 plants	F_2 phenotypes			χ^2 for 3 : 1 ratio	P
			Green	Variegated	Albino		
V ♀ × G ♂	Green	47	681	255	46	17.3	< .01
	Variegated	24	270	89	28	5.56	.01–.05
G ♀ × V ♂	Green	38	1322	347	113	0.60	.3–.5
Backcross							
F_1V(V×G) ♀ ×G♂	Green	7	169	0	0	—	—
	Green	6	93	31	7	1.12	.20–.30
F_1V(V×G) ♀ ×V♂	Green	8	133	45	7	0.96	.3–.5

In the backcross V F_1 ♀ × G ♂ a 1 : 1 ratio of *WW* and *Ww* plants is expected if one gene pair is segregating. Of 13 green backcross plants obtained (Table III), seven bred true for green (Table IV) while six gave, when selfed, progenies approximating the expected 3 green : 1 others (χ^2 for 3 : 1 ratio based on bulked results of backcross F_2 is 1.12).

When V F_1 ♀ are backcrossed to V ♂ all the green offspring are expected to be heterozygous. The eight green backcross plants obtained (Table IV) all gave, when selfed, segregating progenies (χ^2 for 3 : 1 ratio calculated from bulked results is 0.96). The segregation of the backcross hybrids is in good agreement with the monofactorial interpretation.

Almost exactly one-third of the green F_2 plants bred true (Table V), while two-thirds yielded segregating progenies. This result is in accord with the hypothesis that a single gene pair *Ww* is involved. The gene for green appears to be dominant when only "Green" plastids or proplastids are present in the egg.

TABLE V

F_3 POPULATIONS FROM GREEN (G) AND VARIEGATED (V) F_2 PLANTS

Cross	F_1 phenotype	F_2 phenotype	No. of F_2 plants	F_3 phenotypes			F_2 genotype	Deviation from expected F_3 ratio	*P*
				Green	Variegated	Albino			
G♀ × V♂	G	G	133	3164	0	0	*WW*	0	1.0
	G	G	279	3911	1483	176	*Ww*	266.5	<.01
	G	V	49	0	333	568	*ww*	0	1.0
	G	V	3	38	10	12	*Ww*	7	.01–.05
V♀ × G♂	G	G	19	456	0	0	*WW*	0	1.0
	G	G	39	657	224	29	*Ww*	25.5	.05–.30
	G	V	11	0	19	221	*ww*	0	1.0
	G	V	1	22	0	2	*Ww*	4.0	.05–.30

That plastid mutations may occur in heterozygous individuals is indicated by the rather large deviations from expected 3 : 1 ratios in segregating F_3 populations. Actual proportions approach a ratio of 2 green : 1 others in some groups. Four of 64 variegated F_2 plants (6.2%) from green F_1 produced green F_3 progeny and therefore probably had the *W* (green) gene.

Although a good fit to a 12 : 3 : 1 ratio is apparent in some F_2 populations, no similar tendency is observed in the F_3. When all the available data from crosses are considered the most reasonable explanation of the results is that in F_1 hybrids there is one segregating chromosomal factor pair *Ww*; that *W* cannot cause mutations of albino plastids to green but that *w*, even in heterozygous individuals, may cause mutation of some green plastids to white. Distorted ratios are attributed mainly to the irregularities of plastid segregation and transmission.

Albino × Regal (Green) Crosses

Attempts to rear albinos to maturity following Spoehr's (6) method were unsuccessful. Crosses involving white tillers of variegated plants (♀) and Regal (♂) have yielded only four viable seeds, all of which produced albino

seedlings. There is, thus, from these slight results, no evidence of direct plastid transmission by the male.

Discussion

Inheritance of variegation in barley parallels in several respects that of the iojap character in corn (5). In both, plastid mutation occurs in the presence of the homozygous mutant gene. The plastid mutation rate is sufficiently low to permit at least some homozygous but variegated plants to reach maturity and to produce seeds. Mutated plastids do not revert to normal in the presence of the plus gene.

Since the barley plastid mutation is gene-induced, the plastid is to that extent like other cytoplasmic components and unlike most nuclear genes. Few genes are known to induce mutation of other specific genes. An exception is the *Dt* gene in corn, which affects the *a* locus (4).

The mechanism of induction of the plastid mutation is not known, but probably involves the production, by the mutant nuclear gene *w*, of a diffusible substance that induces a specific directed plastid mutation. An alternative suggestion that the mutant gene *w* fails to provide some substance essential for normal chloroplastid development is probably not correct since white plastids do not become green even though the normal green (*W*) gene is present; e.g., in F_1 plants of the V × G cross.

If the albino plastids were immediately responsive to controlling nuclear genes an investigation designed to determine the point at which chlorophyll synthesis is blocked might result in useful information. However, since the albino plastids can presumably be converted to normal only by reverse plastid mutation it would appear useless to search for a chemical substance that, when introduced into white cells, would enable mutant plastids to develop into functional green plastids.

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A CONVENIENT METHOD OF OBTAINING ASCOSPORES FROM BAKERS' YEAST[1]

By A. M. Adams[2]

Abstract

The superiority of methods involving the use of sporulation media containing acetate, first introduced by Stantial and Elder, over several commonly employed methods is established. A new method for obtaining ascospores from bakers' yeast cultures is recommended involving the direct transfer of vegetative cells from a solid nutrient medium to a solid medium containing acetate. High yields of ascospores are consistently produced after seven days' incubation. This method should lend itself particularly to use in the preparation of ascospores for instructional work, and for genetic research in yeast, and may also find application in yeast taxonomy. The technique recommended is as follows: vegetative yeast cells are multiplied on tomato juice agar or on dextrose nutrient agar, and are then transferred to a solid sporulation medium containing 0.04% dextrose, 0.14% anhydrous sodium acetate, and 2% agar.

Introduction

The method most commonly employed to induce ascospore formation in yeast (the term "sporulation" is commonly employed in the literature when referring to this phenomenon) is the "plaster block" or, as it is also called by some workers, the "gypsum block" method. Plaster of Paris is mixed with water and allowed to harden. Vegetative yeast cells are then spread thinly over the surface, which should be kept moist. With some modifications the procedure remains essentially the same as when first introduced by Engel (5) in 1872. Rees (14) in 1869 conducted studies in which he obtained ascospores from vegetative cells after their transfer to a substrate of vegetable tissue. More recently Gorodkowa (6) employed successfully an agar medium containing only a small amount of glucose, beef extract, and sodium chloride. Similarly, McKelvey (12) was able to induce ascospore formation with a weak carrot extract agar. Mrak, Phaff, and Douglas (13) obtained ascospores from agar slant cultures of vegetative cells on a vegetable extract medium. Investigating this problem, Lindegren and Lindegren (11) employed several fruit and vegetable extract agars.

One of the first workers to study sporulation yeast, de Seynes (16) found ascospores were formed when a wine yeast was transferred from grape must of ordinary sugar content to a solution containing only a small amount of sugar. More recently, Wickerham *et al.* (21) have recommended the use of commercial mixed vegetable juices.

Stantial, and later Elder, working in the Department of Chemistry of the University of Toronto have done much interesting and valuable work, the greater portion of which has not been published. Stantial found that a dilute

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A contribution from the Department of Botany, McMaster University, Hamilton, Ont. Based on a thesis submitted to the Department of Botany, McMaster University, in partial fulfillment of the requirements for the degree of Master of Arts, October, 1948.

[2] *Graduate Student.*

medium containing sugar induced sporulation (17), and later (18) that a dilute medium containing both a sugar and an acetate consistently induced sporulation in vegetative cells. She was able to establish an optimum relationship between the concentration of cells and the concentration of acetate in the sporulation medium. She found that as the pH of the sporulation medium was decreased the yield of asci was also reduced. Best yields of ascospores were obtained with pH values of about 8 in sporulation media.

In unpublished manuscripts Elder (2, 3, 4) confirmed and enlarged upon Stantial's findings. As a presporulation medium Elder recommends a broth made by filtering, neutralizing, and autoclaving a commercial brand of tomato juice. Cells cultured in flasks for 24 to 48 hr. in this broth are freed from the liquid by filtration or centrifugation and washed twice with sterile distilled water. The cells are then resuspended in sterile distilled water. After adjustment of the cell concentration with the aid of a haemocytometer, a volume of suspended cells is mixed with an equal volume of liquid sporulation medium, making a final concentration of 20 million cells to 2 mgm. dextrose and 15 to 20 mgm. sodium acetate.

The writer recently had occasion to investigate some of the above methods since he required a reliable source of ascospores for certain research on yeast, and in this connection the method of Stantial and Elder was given special attention.

Materials and Methods

Yeast cultures referred to in the experiments were of the commercial bakers' yeast type. Stock cultures were obtained by means of dilution and plating of cells from packaged yeast. Unless stated to the contrary, the cells transferred to sporulation media were from 24-hr. subcultures.

The media most commonly employed in the experiments to follow were:

(*a*) Dextrose nutrient broth—prepared by adding 5% dextrose to Difco nutrient broth.

(*b*) Dextrose nutrient agar—prepared by adding 2% agar to the broth in (*a*).

Tartaric acid (0.5%) was added to (*a*) and (*b*) to inhibit any bacterial contaminants.

(*c*) Grape juice broth—juice of freshly crushed red grapes was filtered, clarified, and diluted to approximately 5% sugar content.

(*d*) Grape juice agar—prepared by adding 2 to 2.5% agar to (*c*).

(*e*) Tomato juice broth—commercial canned tomato juice (salted) was filtered and clarified.

(*f*) Tomato juice agar—prepared by adding 2% agar to (*e*).

(*g*) Liquid sporulation medium—prepared by dissolving 0.04% dextrose and 0.14% anhydrous sodium acetate in distilled water. This medium is essentially the same as that employed by Elder.

(*h*) Solid sporulation medium—prepared by adding 2% agar to (*g*).

The percentage of ascospores in a given sporulation culture was estimated by counting a total of 400 asci and vegetative cells. Only asci containing two, three, or four ascospores were included in such counts to avoid the possibility of mistaking a vegetative cell for an ascus containing but one ascospore.

During the repeated employment of the Stantial–Elder method for producing ascospores, certain modifications that increased the value of the method were developed. These were:

(*a*) Vegetative cells may be multiplied on a solid nutrient medium instead of in a liquid medium. This obviates the necessity of filtration to free the cells from the culture medium.

(*b*) Similarly, the liquid sporulation medium may be replaced by a solid medium (see (*h*) above). When the latter is employed, cells may be transferred directly to sporulation medium from solid nutrient medium without involving the numerical estimation and adjustment of cell suspensions.

(*c*) When liquid sporulation medium is employed, the concentration of cell suspensions may be adjusted by comparison with permanent density standards prepared and employed as in the estimation of bacterial cell numbers by the McFarland nephelometer technique. This method of estimating cell concentrations was found to be much more rapid than the haemocytometer method employed by Stantial and Elder.

Experimental

(a) Effect of Pretreatment

In this experiment the yeast was grown in nine different media from which the cells were transferred to solid sporulation medium. This transfer was direct except in the case of the liquid medium where filtration was necessary to obtain the cells. The results listed in Table I confirm the findings of

TABLE I

ASCOSPORE FORMATION BY CELLS GROWN ON NINE MEDIA

Pretreatment media	Percentage of ascus formation on solid sporulation medium after:	
	6 days	10 days
Dextrose nutrient agar	80	85
Grape juice agar	55	60
Grape juice	55	65
Tomato juice agar (commercial)	80	85
Tomato juice agar*	66	75
Glucose tryptone extract agar*	60	65
Beef infusion agar*	58	60
Beef lactose agar*	50	50
Nutrient agar*	20	20

* *Difco products prepared according to directions on containers.*

Stantial and Elder that cells grown on tomato juice give high yields of ascospores. Whereas low yields were obtained from those grown on nutrient agar, the other media gave yields comparable to that of tomato juice.

(b) Effect of Certain Chemicals on Ascospore Formation

The ability of nine chemicals to induce ascospore formation in sugar-free liquid medium was assessed. Five concentrations of each chemical were employed and each sporulation culture had a volume of 20 ml. containing one million yeast cells per ml. Erlenmeyer flasks of 250 ml. capacity were employed as containers for the 20 ml. volumes of sporulation culture. (Throughout the remainder of the experimental work these containers were used for liquid sporulation medium, unless otherwise specified.) Vegetative cells for use here were grown on dextrose nutrient agar medium. At the conclusion of the experiment, viability of the cultures was determined by standard plating procedure to determine whether the chemicals had been toxic to the cells.

Table II indicates that the acetate radical was responsible for inducing ascospore production. Higher yields of ascospores were obtained from cultures in which the concentration of acetate was that recommended by

TABLE II

THE EFFECT OF VARIOUS CONCENTRATIONS OF CHEMICALS UPON ASCOSPORE FORMATION

Compounds employed*	Percentage yield of asci					Viability
	0.185**	0.375**	0.75**	1.5**	3.0**	
Potassium acetate	70	68	65	68	68	+
Calcium acetate	72	58	48	41	41	+
Acetic acid	65	25	0	0	0	+
Barium acetate	0	0	0	0	0	Much reduced
Sodium acetate	78	77	64	57	35	+
Magnesium acetate	65	65	32	8	0	+
Sodium oxalate	0	0	Rare	0	0	+
Sodium citrate	0	0	Rare	0	0	+
Calcium sulphate	0	0	0	0	0	+

* *All chemicals were C.P. grade.*

** *These figures represent the percentage composition of the anion in 100 ml. of the sporulation medium.*

Elder (4). Yields obtained from sodium acetate were equal to or greater than those from any other compound tested. The failure to obtain ascospores with barium acetate may have been due to a toxic effect since the viability of the cells was found to be greatly reduced. Strontium acetate was tested in another experiment and proved highly effective at inducing ascospore formation with both liquid and solid medium. Stantial (18), in an effort to

find agents that would induce ascospore formation, used many different chemicals. She obtained the best yields from sodium and potassium acetates—the two acetates reported in her studies.

(c) Effect of Cell Numbers in Liquid Sporulation Medium on the Yield of Ascospores

Sporulation cultures were prepared in which the concentrations of acetate and dextrose were constant and in the proportions described in the section on Materials and Methods. The number of cells in each culture was varied so that the effect of various cell populations on sporulation could be assessed. Two cultures of commercial yeast were used. The percentages of asci present after 14 days' incubation are listed in Table III. The percentage yield of

TABLE III

THE EFFECT OF CELL NUMBERS IN LIQUID SPORULATION MEDIUM ON YIELD OF ASCOSPORES

Millions of cells per 20 ml. of liquid sporulation medium	Percentage yield of asci after 14 days	
	Culture No. 1	Culture No. 2
5	2	66
10	37	60
20	40	70
40	44	30
80	25	10
160	2	3

asci in culture No. 1 was reduced above and below the concentration of 20 million cells in 20 ml. of sporulation medium. This was repeatedly shown to occur by Elder (4). But in culture No. 2 the percentage of asci did not drop appreciably in the lower concentrations, indicating that yeast strains vary in this respect.

(d) Effect of Depth of Medium on Ascus Formation

Ascospore formation was observed to take place readily in culture suspensions in 250 ml. flasks when the volume of the suspensions was 20 ml. or less, but when similar suspensions were incubated in 10 ml. amounts in test tubes very few ascospores developed.

Investigating further, varying amounts of a sporulation medium cell suspension were added to a series of test tubes (16 mm. × 150 mm.). As shown in Table IV, from tubes in which the depth of the medium was 9 mm. or less, a high yield of ascospores was obtained, whereas at greater depth the percentage yield rapidly decreased. The factor responsible for this effect has as yet not been determined.

TABLE IV

THE EFFECT OF DEPTH OF MEDIUM IN CULTURE TUBES ON ASCUS FORMATION

Depth of liquid sporulation medium, cm.	Volume of liquid sporulation medium,* ml.	Percentage of asci formed
0.55	0.25	84
0.70	0.50	76
0.90	0.50	70
1.00	1.00	5
1.05	1.00	2
1.70	2.00	2
2.00	2.00	2
2.60	3.00	0
3.20	4.00	Rare
4.60	6.00	Rare
6.10	8.00	0
6.90	10.00	0

* *The exposed surface area in each instance was 2.0 cm².*

(e) Effect of Removing Cells from Sporulation Medium Containing Acetate to Distilled Water

Stantial (18), and Elder (4), found that cells first suspended in the sporulation medium for four to six hours, then washed and resuspended in water, yielded asci. This matter was further investigated here in an experiment designed to determine how short an exposure to acetate–dextrose medium is required to induce ascospore formation. Cells were suspended in the acetate–dextrose sporulation medium for varying intervals, washed and resuspended in distilled water, then incubated. The asci present were estimated after seven days' and 14 days' incubation. No appreciable difference in the yields observed on the two days was noted. Cells suspended in the medium for periods of one-half, one, two, three, four, and five hours gave yields of 9, 7, 11, 13, 12, and 14% asci, respectively. The three control flasks whose cells remained in the acetate–dextrose mixture throughout the incubation period gave an average yield of 60%.

(f) Ascus Production by Cells from Cultures of Various Ages

A comparison between the agar to liquid transfer method and the agar to agar transfer method was made with cultures from dextrose nutrient agar of a commercial baking yeast 1 day, 12 days, 22 days, and 36 days old.

Table V shows that the older cultures failed to produce as high a percentage of asci as did the young culture. Perhaps this may be explained by the fact that in the older cultures a greater number of dead or heavily granular cells would be present. The ascospores produced were in all instances normal in appearance, the asci being observed to contain two, three, or four ascospores. It is clear from this experiment that to obtain maximum yields of ascospores it is preferable to use fairly young cultures of vegetative cells. Elder (4) showed that cells from young cultures gave the most satisfactory yields.

TABLE V

THE PERCENTAGE OF ASCUS PRODUCTION BY CELLS OF VARIOUS AGES EMPLOYING SOLID AND LIQUID SPORULATION MEDIA

Age of vegetative cells	Solid sporulation medium			Liquid sporulation medium		
	Percentage of asci after:					
	6 days	10 days	14 days	6 days	10 days	14 days
1-day-old culture	62	65	75	56	60	60
12-day-old culture	22	20	25	10	15	15
22-day-old culture	20	20	20	15	13	15
36-day-old culture	5	5	5	Occ.	Occ.	Occ.

Incidentally, it will be noted in Table V that, in all instances, higher yields were obtained with solid than with liquid sporulation medium.

(g) Comparison of Sporulation Media Containing Acetate

In this experiment the complete Stantial–Elder method was compared with the following modifications: (1) cells were multiplied on solid nutrient medium and added to liquid sporulation medium, (2) cells were multiplied on liquid nutrient medium and added to solid sporulation medium, and (3) cells were multiplied on solid nutrient medium and added to solid sporulation medium. It is seen that (1) embraces modification (*a*) as described under Materials and Methods, (2) embraces modification (*b*), and (3) both (*a*) and (*b*).

The yields listed in Table VI show that these modifications when employed give yields quite comparable to those obtained with the Stantial–Elder

TABLE VI

THE EFFECT OF VARIED TECHNIQUE UPON THE FORMATION OF ASCOSPORES WITH SPORULATION MEDIA CONTAINING ACETATE

Medium on which cells were multiplied	Cells transferred to solid sporulation medium			Cells transferred to solid sporulation medium containing a trace of peptone			Cells transferred to liquid sporulation medium		
	Percentage of asci after:								
	6 days	10 days	14 days	6 days	10 days	14 days	6 days	10 days	14 days
Dextrose nutrient agar	70	72	80	85	80	90	50	64	60
Grape juice agar	75	75	80	85	83	90	66	70	72
Tomato juice agar (Difco)	70	74	80	80	85	85	63	66	70
Tomato juice agar (commercial)	73	70	75	80	88	90	72	68	73
Grape juice	62	65	65	70	70	75	50*	57*	60*

* *Complete Stantial–Elder technique employed.*

technique. The addition of a trace of peptone to the solid sporulation medium gave the highest yields. The five types of media employed to multiply the vegetative cells had no great effect upon the yield of ascospores. This confirms the results in the experiment on pretreatment effects described under (*a*) in which these media were also employed.

(h) Comparison of Methods Generally Employed for Inducing Ascospore Formation

Cultures were prepared according to methods recommended by different authors and at present in general use for the development of ascospores. The yields of asci obtained in successive trials by these methods and the method developed in this work were averaged and are tabulated for comparison in Table VII.

TABLE VII

THE RELATIVE FREQUENCY OF ASCI FORMED BY VEGETATIVE CELLS IN VARIOUS ENVIRONMENTS RECOMMENDED FOR INDUCING ASCOSPORE FORMATION

Sporulation medium or substratum	Percentage of asci present after 10 days at room temperature
Gorodkowa	20
McKelvey	2
Gypsum block	<1
Gypsum block and peptone water	<1
Carrot slab	3
Liquid sporulation medium	75
Solid sporulation medium	85
Solid sporulation medium with added peptone	90

The medium developed by Gorodkowa (6) contains only a small amount of glucose, beef extract, and sodium chloride in an agar base, thus providing a very limited supply of nutrients for the vegetative cells. This medium as is shown in Table VII ranked next to those containing acetate and was much more effective as a sporulation medium than the carrot slab, the gypsum block, or the weak carrot infusion medium of McKelvey (12). Figs. 7 and 8 show the development of ascospores upon solid sporulation medium containing acetate after four days' and 14 days' incubation. Many vegetative yeast cells can be seen in Fig. 7, whereas in the 14-day culture (Fig. 8) very few are encountered. Fig. 9 shows the appearance of asci and ascospores from solid sporulation medium under dark-field illumination.

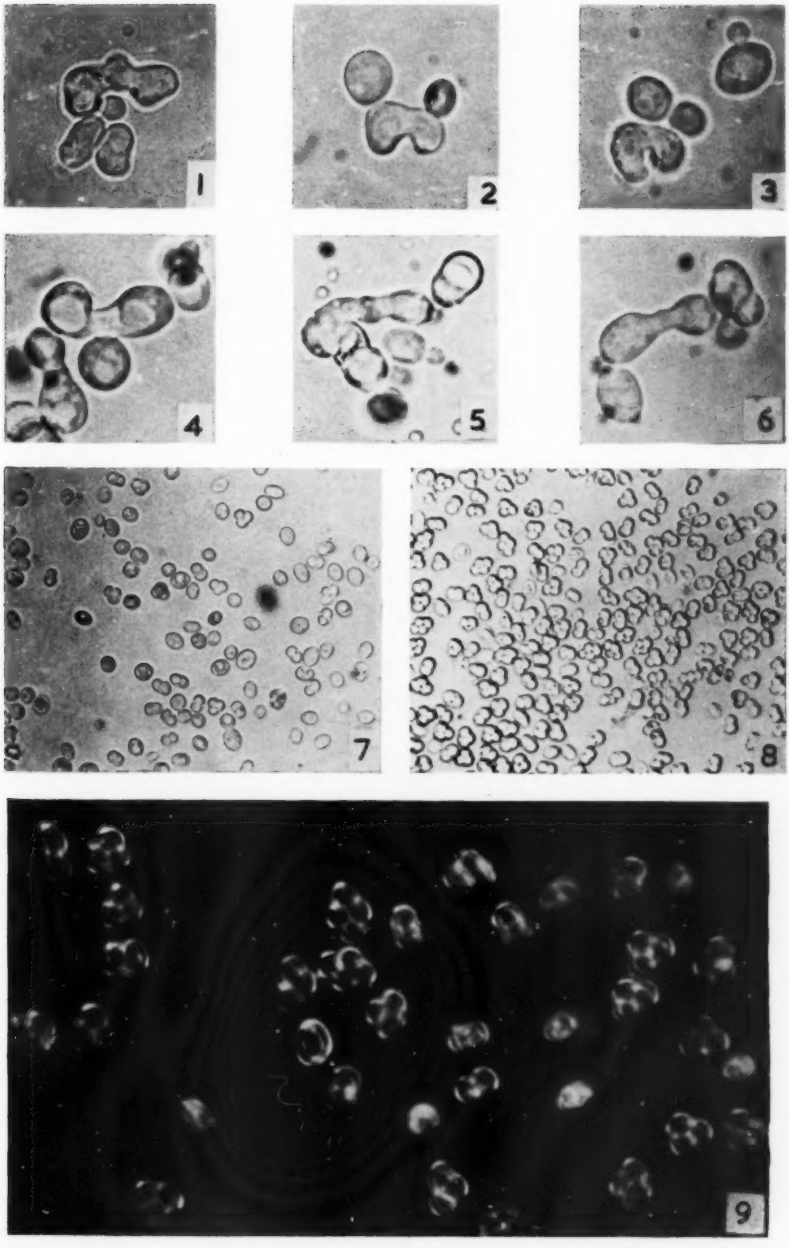
FIGS. 1 to 6. *Microphotograph of cells from mating medium in which ascospores were placed showing evidence of fusions occurring between ascospores and/or vegetative cells.* × *1200.*

FIG. 7. *Ascospore formation on solid sporulation medium after four days.* × *450.*

FIG. 8. *Ascospore formation on solid sporulation medium after 14 days.* × *450.*

FIG. 9. *Asci and ascospores after 14 days on solid sporulation medium. Dark-field illumination.* × *3000.*

PLATE I



Although Petri dishes were found to be quite satisfactory as containers for the solid sporulation medium, test tubes afford a greater measure of protection from contamination during handling or storing. The preparation of sporulation cultures upon slants made with solid sporulation medium was found to give the same high yields of ascospores as did the Petri dish sporulation cultures. Test tube sporulation cultures have been previously prepared by Graham and Hastings (7) employing gypsum slants.

(i) Observations on Fusions between Ascospores

Winge (22) and Lindegren (10) have stated that fusions may occur between ascospores within an ascus, between haploid cells arising from different ascospores, and between ascospores and haploid cells, all three types of fusion resulting in the production of diploid cells.

Experiments were conducted to determine whether the ascospores formed in the presence of acetate would behave in a manner similar to that described by Winge and Lindegren. Employing a mating medium recommended by Lindegren (10) suspensions of ascospores (obtained from solid sporulation medium) were observed at intervals during a period of 24 hr. After 15 hr. a few fusions between ascospores were noted. During the next few hours these became somewhat more numerous, but the situation was confused by the vegetative budding of the cells in the suspension. Several typical fusions are illustrated in Figs. 1 to 6. It seems obvious that the ascospores formed by the method recommended here behaved in a like manner to that observed by Winge among ascospores produced on gypsum blocks.

Discussion

Most of the methods recommended in the literature for inducing ascospore formation involve the use of plant tissue or tissue extracts. While it is possible that small quantities of acetate may have been present in these plant extracts, the first worker to recommend the addition of acetate was evidently Dr. Stantial. In 1939, Baltatu (1) working with the gypsum block method found that addition of acetic acid to the dilute grape juice medium that he poured over gypsum blocks increased the yield of ascospores.

But other factors than the presence of acetate may operate to stimulate the production of ascospores. Tanner (19) expressed agreement with Hansen (8) that a plentiful supply of air and a negligible amount of nutrient are required for good sporulation. Salle (15) states that sporulation can be brought about by culturing cells on starvation medium. Welten (20) spread washed yeast cells over glass plates and observed sporulation. It thus seems that adverse environmental conditions stimulate ascospore formation, which may account for the success of the plaster block method. On the other hand, Stantial was able to induce sporulation by adding orange, lemon, grapefruit, or tomato juice, and the juice expressed from lettuce to washed yeast cells in a water suspension. Lindegren and Lindegren (11) also had success with various combinations of vegetable and fruit juices. Evidently ascospores may be

formed in environments not unfavorable to growth. Thus from the literature it is evident that the environmental factors responsible for the formation of asci in yeast are not as yet fully understood.

A convenient and reliable method of obtaining ascospores is of some practical importance. Ascospores are essential for hybridization research leading to the development of improved yeast strains for industrial purposes. Also, since the characteristics of the ascus are of fundamental importance in the classification of yeasts, such a method would be of great value in identification of unknown strains. Henrici (9) recommends the plaster block method with McKelvey's medium (12) and Gorodkowa's (6) as alternative methods but cautions that, "Spore formation with yeasts is, however, always uncertain, and a strain should not be considered non-sporogenous until several trials have been made with different methods". Lindegren (10), in a recent publication, studied the ability of 40 commercial bakers' yeasts to form ascospores. Here the highest yield reported was 50% asci; and of the remainder, 85% gave yields below 30%. Of these, 12% produced less than 1% of asci. The behavior of Lindegren's cultures in an acetate sporulation medium would be most interesting, as would also be the behavior of representatives of the *Torula* and *Zygosaccharomyces* yeasts. The possible effect that this improved method of obtaining ascospores may have upon yeast classification appears worthy of investigation.

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